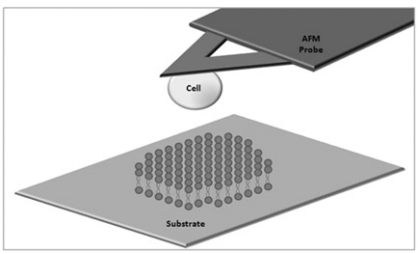
We explore the ultrastructure of the well-studied *Drosophilamelanogaster-gypsy* chromatin insulator by immunolabeling a key insulator protein CP190 using a fluoronanogold conjugated antibody probe. In our correlative method, fluorescent imaging is initially performed to identify nuclei that contain insulator bodies, which are rare within thin sections. A comparison of low-magnification EM image of a whole cell with the corresponding fluorescent image reveals the approximate location of the structure of interest. The fluorescence signal observed by light microscope guarantees the presence of the conjugated nanogold, which can be visualized using STEM, and used to locate precisely the labeled CP190 proteins. EFTEM is then performed to image the distribution of nitrogen and phosphorus and thus map the distributions of protein and nucleic acid. It is evident from these two elemental maps that the insulator body contains an abundance of protein but a small quantity of nucleic acid. Even though dense chromatin surrounds the insulator body, it is difficult to determine whether the low levels of phosphorus within the insulator body structures correspond to DNA or RNA, which requires further investigation.

3333-Pos Board B438
Dominant Vinculin Binding Angle in Podosomes Revealed by High Resolution Optical Microscopy
Susan Cox, Marie Walde, James Monypenny, Rainer Heintzmann, Gareth Jones.
Podosomes are dynamic actin-rich cell-matrix adhesion sites of migrating and invasive cells such as macrophages and osteoclasts, and are receiving increasing attention due to their possible involvement in physiological events such as monocyte extravasation and tissue transmigration, as well as pathological conditions such as atherosclerosis, osteoporosis and cancer metastasis. These structures were examined using three different fluorescence microscopy techniques which provide resolution below the diffraction limit: structured illumination microscopy, stimulated emission depletion microscopy and stochastic optical reconstruction microscopy have been used. In high resolution images, it is clearly visible that each podosome consists of an actin core surrounded by a protein-enriched ring, supporting the existing podosome model. However, these rings are polygonal structures rather than smooth circles. An analysis of the binding angles at corners reveals vinculin to have a dominant binding angle of around 115 degrees.

3334-Pos Board B439
Imaging Fluorescence Cross-Correlation Spectroscopy as a Tool to Study Cell-Membrane Organization
Jagadish Sankaran, Nirmalya Bag, Thorsten Wohland.
The structure of biological membranes has been investigated for many years. However, progress is hindered by the fact that putative domains are highly dynamic and their size is smaller than the optical diffraction limit and thus direct observations are difficult. Therefore, there is a need to develop new biophysical tools which can infer the existence of domains within membranes and can follow their development over time. We have introduced in the past a method called Imaging Total Internal Reflection-Fluorescence Correlation Spectroscopy (ITIR-FCS) using EMCCD or sCMOS cameras. ITIR-FCS allows the measurement of a large number (up to ~0.5 million) correlation curves at contiguous locations on cell membranes of live cells with millisecond time resolution. The spatial information within the data can be used to obtain information on the structure and organization of the membranes. This is achieved by calculating differences between the forward and backward cross-correlations between adjacent pixels A and B (CCF_{AB} - CCF_{BA}) or A, B, and C (CCF_{AB} - CCF_{CB}). The results can be depicted as histograms referred to as ΔCCF distributions. In this work we conduct measurements on supported lipid bilayers and cell membranes and perform simulations to demonstrate how ΔCCF distributions change characteristically with membrane complexity and structure. In particular, we demonstrate that domains with sizes below the diffraction limit have a characteristic broadening effect on the ΔCCF distributions. As an example we show that changes in membrane structure and organization of live neuroblastoma cells can be followed over the time course of an hour or more by way of ΔCCF distributions. To deal with large amount of data collected we developed an open source software, ImFCS, to calculate and fit the auto- and cross-correlation functions and depict the results in an imaging format.

3335-Pos Board B440
Probing Orientational Order of MHC Class I Protein and Lipids in Cell Membranes by Fluorescence Polarization-Resolved Microscopy Imaging
Alla Kress, Hubert Ranchon, Patrick Ferrand, Hervé Rigneault, Sophie Brasselet, Tomasz Trombik, Hai-Tao He, Didier Marguet.
Biomolecular orientational organization of lipids and proteins in plasma membrane is a crucial factor in biological processes where functions can be closely related to orientation and ordering mechanisms. The concept of transient nanosized phase separations in ordered and disordered domains, called ''lipid rafts'' is now widely accepted. Furthermore, the ordered domains are enriched in signalling proteins, which highlights the crucial impact of phase separation during the signalling processes. While this field has been so far largely addressed by studying the translational diffusion behaviour of membrane proteins by Single Molecule Tracking or Fluorescence Correlation Spectroscopy, only little is known about the orientational behaviour of signalling proteins in plasma membranes, mainly due to the lack of appropriate rigid fluorescent label which would be able to act as a proper orientation reporter. In this work we develop a fully polarization-resolved fluorescence imaging technique using a tuneable incident polarization state (''fluorescence polarimetry''), in combination with fluorescence anisotropy imaging, in order to provide orientational order information in very general cell membranes shapes.
We apply this technique to the measurement of quantitative orientational distribution of MHC Class I proteins in the plasma and nuclear membranes, benefiting from a rigidly attached GFP probe. The surrounding lipid orientational order in the plasma membrane is additionally probed using the fluorescent reporter di-8-ANEPPQ.The MHC Class I protein is found to be more ordered in the plasma membrane as compared to the nuclear membrane. Both MHC I and di-8-ANEPPQ orientational orders in the plasma membrane are furthermore seen to be highly affected by actin depolymerisation upon Latrunculin A treatment, with variations that indicate both a structural change in the membrane morphology and a disruption of MHC I - actin interactions.

3336-Pos Board B441
A Combined Confocal-Total Internal Reflection Fluorescence (TIRF) Single-Cell Microscopy Investigation of CEACAM1 Dynamics
Laura N. Poloni, Christopher M. Yip.
The carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) represent a subset of the immunoglobulin superfamily of cell adhesion molecules that mediate intercellular adhesion. One member of this subset, CEACAM1, is particularly interesting since it is down-regulated in tumourigenic cell lines. CEACAM1 exists as an equilibrium between monomers and dimers; however, the spatial and temporal distribution upon cell-substrate contact is not known.
In order to determine CEACAM1 dynamics, a coupled confocal-total internal reflection fluorescence (TIRF)-atomic force microscopy (AFM) platform was used. Live YFP-CEACAM1 labelled cells were isolated on AFM tips for controlled positioning of cells. Confocal microscopy was used to map CEACAM1 receptors over the entire cell surface. Coupled TIRF microscopy monitored the free cell surface as it was brought into contact with a substrate (i.e. another cell, a model membrane, modified glass). Confocal and TIRF microscopy homoFRET measurements were used to determine the distribution of monomeric and dimeric CEACAM1 receptors prior to and upon cell-substrate contact. Through this understanding of how molecular organization affects intercellular binding and signal transduction, it may be possible to identify peptide or pharmacological drug strategies to create CEACAM-focused therapies for cancer.



3337-Pos Board B442
Following Actin Fibers in 3D During Cell Migration in Collagen Matrices
Michelle A. Digman, Chi-Li Chiu, Jose S. Aguilar, Enrico Gratton.
Actin polymerization is a major mechanism for the production of the force necessary for cell migration in 2D. The polymerization of actin and its retrograde motion at the leading edge of cell moving in 2D has been studied in great detail as well as the interaction of actin with focal adhesions. When cells grow in 3D collagen matrices, the extending lamellipodial protrusion is more difficult to visualize and it is likely not relavent for the movement of the cell over large distances. We use the modulation tracking 3D method to accurately image the cell protrusion. This method is capable of producing detailed images of 3D structures at the nanoscale and at the same time measure diffusion and aggregation of molecules in these structures. In 3D, cells produce very long protrusions that presumably grab on the surrounding collagen fibers to propel the rest of the cell

body in a specific direction. What is unclear is if the cell is capable of forming stable adhesions with the collagen matrix and how the force is generated. Using the modulation tracking imaging method we can follow the changes in shape of the cellular protrusion and also image separately various proteins, including actin in the cytoplasm and in the membrane. In the thin long protrusion we observe both fast diffusing actin molecules and also relatively immobile species, presumably part of the actin cytoskeleton. We are developing a method to directly measure the movement of the entire actin bundle inside the very thin cells protrusions. The method is conceptually similar to speckle imaging; however, it works in 3D.

3338-Pos Board B443

Super-Resolution Imaging of Chromosomal DNA in Cells

Paul D. Simonson, Eli Rothenberg, Paul R. Selvin.

Super-resolution imaging is achieved by localizing diffraction-limited spots with high accuracy. Here we combined two powerful approaches to image the chromosomal DNA inside cells. In one method, the accumulated, stochastic binding of fluorescent labels to an imaging target are localized, while in the other the fluorophore transitions between dark and bright states (compatible with binding, photobleaching, photo-activation, blinking, etc.), even when fluorophore images overlap, are localized. Combining the two techniques results in a robust microscopy that is faster than what is possible with either technique alone, requires less optimization, and corrects for cell autofluorescence. In addition, background noise due to fluorescent labels in solution can be virtually eliminated by using labels that fluoresce only when bound to the target.

Many DNA-specific dyes show dramatic fluorescence enhancement upon binding to DNA, including SYTO, LOLO, and YOYO dyes. We used nanomolar concentrations of SYTO and LOLO to image lambda DNA attached to poly-L-lysine coated glass and chromosomal DNA in fixed HEK 293 and HeLa cells. We found average single-fluorophore localization errors of 36 nm and 24 nm on glass and in cells, respectively. These imaging techniques may prove useful in future studies of chromosomal DNA in cells, including chromatin structure and defects.

This approach was further applied to imaging microtubules in vitro. We used commercial Oregon Green 488 paclitaxel to achieve a 10 nm average fluorophore localization error, and streptavidin S45A to transiently label biotinylated microtubules with Atto647N, resulting in an average of 18 nm fluorophore localization error. Future work will involve simultaneous imaging of DNA and proteins to answer important biological questions.

3339-Pos Board B444

Investigation of Lysosomes as Enzyme Storage Vesicles using Single Particle Tracking Fluorescence Microscopy

William H. Humphries IV, Christine K. Payne.

Intracellular, vesicle-mediated, degradation of extracellular cargo is an essential cellular function. Of particular interest is the population of vesicles responsible for degradation of extracellular cargo. Previous work using low-density lipoprotein (LDL), a classic extracellular cargo, demonstrates that the enzyme-mediated degradation of LDL occurs in a hybrid late endosomal-lysosomal vesicle[i]. In addition, the degradation of LDL occurs within 60 s of the colocalization of LDL with a lysosomal protein, LAMP1. These observations suggest that lysosomes are responsible for delivering enzymes to late endosomes, forming a hybrid organelle in which degradation occurs. Using single particle tracking fluorescence microscopy, we investigate the hypothesis that lysosomes serve as enzyme storage vesicles. We test this hypothesis using two different approaches. The first approach uses cell-permeable drugs that inhibit specific enzymes known to degrade LDL; CA074ME, a cathepsin B inhibitor, and pepstatin methyl ester, a cathepsin D inhibitor. The second approach uses siRNA to knockdown expression of LAMP. Unique to these experiments is the ability to directly monitor, in intact live cells, LDL degradation utilizing a fluorescence labeling scheme that reports on the integrity of the LDL particle. This method involves conjugation of distinct fluorophores to the protein and lipid components of LDL. Upon degradation the protein reporter decreases in fluorescence intensity while the lipid reporter increases fluorescence intensity. Our experiments make it possible to describe the complete endocytic pathway of LDL from internalization to degradation and provide a more complete picture of the intracellular degradation of extracellular cargo.

[i] W.H. Humphries IV, N.C. Fay, and C.K. Payne, *Intracellular degradation of low-density lipoprotein probed with two-color fluorescence microscopy*, Integrative Biology, in press, (2010).

3340-Pos Board B445

Superresolution Imaging of Intact Microbial Communities Reveals Molecular Architecture of Biofilm Development and Bacterial Organization

Veysel Berk, Nicholas Fong, Graham Dempsey, Omer Develioglu, Xiaowei Zhuang, Fitnat Yildiz, Steve Chu.

Most bacteria live as a biofilm community in their natural habitat. This surface-attached social life form is commonly found in antibiotic-resistant infections and chronic diseases. For example, bacterial biofilms are a leading cause of lung infection and death among cystic fibrosis patients. Biofilms are also crucial for bioenergy research since cellulose degrading bacteria in the gut of termites are organized as heterogenic biofilms and believed to communicate throughout these tissue-like structures. To gain structural and molecular insight on biofilm formation, we imaged intact bacterial biofilms at different developmental stages without using any fixing agents by STORM microscope. These three-dimensional superresolution images revealed ten to twenty biofilm-promoting exopolysaccharide-rich regions that are sparsely distributed on the cell surface. A few hours after surface attachment of bacteria, these small globular structures expanded to ~100 nm in size and protruded from the cell surface. During the initial stage of biofilm formation, we observed extensive interactions among neighboring cells through these globular exopolysaccharides. Moreover, we identified straight cable-like cell-to-cell and cell-to-substrate connections, up to 5 microns in length, originating from globular structures. These physical interactions may explain how bacteria form initial microcluster on the surface, first stage of commitment to biofilm formation. Microcluster formation depends on bacterial twitching motility on the surface and exopolysaccharide synthesis.These results are shifting the paradigm in the biofilm field which states that bacteria are randomly embedded in an extracellular matrix in biofilms. Our data suggests that bacteria actively build their house similar to a spider web by synthesizing sticky globular polysachharides on the cell surface, which are then extended to cable-like structures by twitching motility on the surface.

3341-Pos Board B446

3D Tracking of Single Fluorescent Particles with Sub-Millisecond and Nanometer Resolution

Joerg Bewersdorf, Manuel F. Juette.

Observing dynamics at the nanoscale requires sub-millisecond time resolution. Notably, in studying biological systems, three-dimensional (3D) trajectories of fluorescently labeled objects such as viruses or transport vesicles often need to be acquired with high temporal resolution.

Here, we present a novel instrument (1) which combines scanning-free multiplane detection at 3.2 kHz frame rate and single photon sensitivity with optimized beam-steering capabilities. This setup enables ultrafast 3D localization with sub-millisecond time resolution and nanometer localization precision. We demonstrate 3D tracking of single fluorescent particles at speeds of up to 150 nm/ms over several seconds and large volumes. By focused excitation of only the particle of interest - while avoiding confocal pinholes - the system realizes maximum detection efficiency at minimal laser irradiation. These features, combined with the avoidance of stage movement, provide high live-sample compatibility for future biomedical applications.

Next to the characterization of the instrument, we will show first biomedical applications.

(1) Juette, M.F. and Bewersdorf, J., Nano Letters, in press

3342-Pos Board B447

Fluorinated Membrane Potential Probes

Ping Yan, Adrian Negrean, Huibert D. Mansvelder, Leslie M. Loew.

To explore the effect of fluorination on photophysical properties of membrane potential probes, we developed synthetic methods for ANEP dyes with fluorine substitutions at the donor, bridge, and acceptor sides. Fluorination on acceptor side induces red shifts in optical spectra while on donor side it induces blue shifts. The trend can be qualitatively rationalized as electron redistribution from donor in the ground state to acceptor in the excited state, and quantitatively predicted by quantum mechanical calculations using time-dependent density functional theory (TDDFT). Compared with parent ANEP dyes, fluorinated dyes generally show improved photostabilities in addition to similar fast response kinetics and high voltage sensitivities when tested in a voltage-clamped hemispherical lipid bilayer (HLB) apparatus. The characteristics of red-shifted absorption spectra, improved photostability, and high voltage sensitivity have enabled us to achieve a single trial resolution of 50 mV in two-photon imaging of cultured hippocampal neurons using 1100-1350 nm laser excitation, paving the way for in vivo imaging of spontaneous action potentials. (Supported by NIH grants EB001963).